Relation Between Antioxidant Enzyme Gene Expression and Antioxidative Defense Status of Insulin-Producing Cells

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Antioxidant enzyme expression was determined in rat pancreatic islets and RINm5F insulin-producing cells on the level of mRNA, protein, and enzyme activity in comparison with 11 other rat tissues. Although superoxide dismutase expression was in the range of 30% of the liver values, the expression of the hydrogen peroxide–inactivating enzymes catalase and glutathione peroxidase was extremely low, in the range of 5% of the liver. Pancreatic islets but not RINm5F cells expressed an additional phospholipid hydroperoxide glutathione peroxidase that exerted protective effects against lipid peroxidation of the plasma membrane. Regression analysis for mRNA and protein expression and enzyme activities from 12 rat tissues revealed that the mRNA levels determine the enzyme activities of the tissues. The induction of cellular stress by high glucose, high oxygen, and heat shock treatment did not affect antioxidant enzyme expression in rat pancreatic islets or in RINm5F cells. Thus insulin-producing cells cannot adapt the low antioxidant enzyme activity levels to typical situations of cellular stress by an upregulation of gene expression. Through stable transfection, however, we were able to increase catalase and glutathione peroxidase gene expression in RINm5F cells, resulting in enzyme activities more than 100-fold higher than in nontransfected controls. Catalase-transfected RINm5F cells showed a 10-fold greater resistance toward hydrogen peroxide toxicity, whereas glutathione peroxidase overexpression was much less effective. Thus inactivation of hydrogen peroxide through catalase seems to be a step of critical importance for the removal of reactive oxygen species in insulin-producing cells. Overexpression of catalase may therefore be an effective means of preventing the toxic action of reactive oxygen species.
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Reactive oxygen species play an important role in inflammatory and autoimmune diseases, reperfusion injury, cancer, and diabetes mellitus (1). The process through which pancreatic β-cells are damaged during autoimmune attack is a complex one that includes the effects of cytokines and nitric oxide as well as reactive oxygen species (2–9). Reactive oxygen species cause direct cellular damage by oxidizing nucleic acids, proteins, and membrane lipids (10). On the other hand, it has been proposed that reactive oxygen species mediate the activation of genes involved both in cellular defense and cellular damage (11–14). The extent to which the various tissues contain antioxidative enzymes may be a major determinant for their individual susceptibility to cytotoxic damage. To assess the extent to which pancreatic β-cells contain antioxidant enzymes, we analyzed the expression and regulation of superoxide dismutase (SOD), catalase, and glutathione peroxidase (Gpx). When compared with 11 other rat tissues, pancreatic islets and RINm5F insulin-producing tissue culture cells demonstrated the weakest antioxidant enzyme defense system, particularly with respect to hydrogen peroxide–inactivating enzymes. We therefore overexpressed catalase and Gpx in RINm5F insulin-producing cells, thereby drastically improving cellular defense against reactive oxygen species. Thus the lack of an efficient hydrogen peroxide–inactivating enzyme equipment in pancreatic β-cells may explain the extraordinary sensitivity of pancreatic β-cells to toxic damage during development of autoimmune or chemical diabetes.

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Gpx, glutathione peroxidase; ECL, enhanced chemiluminescence; KRBB, Krebs-Ringer bicarbonate buffer; LDH, lactate dehydrogenase; MTT, dimethylthiazoldiphenyl tetrazolium bromide; NBT, nitro-blue-tetrazolium; SOD, superoxide dismutase.

RESEARCH DESIGN AND METHODS

Materials. Restriction enzymes, the SP6/T7 Transcription Kit, and the DIG Nucleic Acid Detection Kit were obtained from Boehringer (Mannheim, Germany). Hybond N nylon membranes were obtained from Amersham (Braunschweig, Germany) and Immobilon-P PVDF membranes from Millipore (Bedford, MA). Cu/Zn SOD (from bovine liver), Gpx (from bovine erythrocytes), xanthine oxidase, nitro-blue-tetrazolium (NBT), and peroxidase-labeled anti rabbit-IgG antibody were from Sigma (St. Louis, MO). The enhanced chemiluminescence (ECL) detection system and autoradiography films were from Amersham (Braunschweig, Germany), and the guanidine thiocyanate was from Fluka (Neu-Ulm, Germany). All other reagents of analytical grade were from Merck (Darmstadt, Germany). The cDNAs coding for rat cytoplasmic Cu/Zn SOD, rat mitochondrial Mn SOD, and rat Gpx were provided by Dr. Y-S. Ho (Detroit, MI). The cDNA coding for porcine phospholipid Gpx was provided by Dr. R. Brigelius-Flohe (Potsdam, Germany). The human catalase cDNA was obtained from the American Tissue Culture Collection (Rockville, MD). Antibodies against rat Cu/Zn SOD, Mn SOD, and Gpx were provided by Dr. K. Asayama (Yamanashi, Japan). The antibody against bovine catalase was purchased from Rockland (Gilbertsville, PA), the monoclonal antibody against human Hsp70 from Biotrend (Fremont, CA), and the antibody against rat liver heme oxygenase-1 from StressGen (Victoria, BC, Canada). All tissue culture equipment was from GIBCO Life Technologies (Gaithersburg, MD).

Animals and tissue isolation. Fed male Wistar rats (250–300 g body weight) were killed by decapitation. After isolation, tissues were washed in ice-cold phosphate-buffered saline and homogenized in precooled buffer for isolation of RNA and subcellular fractions. Pancreatic islets were isolated by a collagenase digestion procedure using a Ficoll gradient centrifugation for purification. Islets were either used immediately for isolation of RNA and subcellular fractions or transferred to tissue culture for further studies.

Northern blot analyses. Isolated pancreatic islets (n = 200) were homogenized in 300 µl precooled buffered 4 mol/l guanidine thiocyanate solution. The other

tissues were homogenized in the same solution. Total RNA was isolated by a combined water-saturated phenol-chloroform-isoamyl alcohol extraction according to Chomczynski and Sacchi (15), with an addition of ultrapure glycogen to achieve full precipitation of islet RNA. Next 5 μg total RNA per lane was subjected to electrophoresis on denaturing formamide/formaldehyde 1% agarose gels and transferred to nylon membranes. The cDNAs coding for rat cytoplasmic Cu/Zn SOD (16), rat mitochondrial Mn SOD (17), rat Gpx (18), porcine phospholipid Gpx (19), and human catalase (20) were subcloned in the pBluescript SK(+) vector (Stratagene, La Jolla, CA) for the generation of cRNA probes. Hybridization was performed at 68°C overnight in a solution containing 50% deionized formamide, 5 × SSPE (SSPE = 180 mmol/l sodium chloride, 1 mmol/l EDTA, and 10 mmol/l NaH_2PO_4; pH 7.4), 10× Denhardt's solution, 0.5% SDS, 100 μg/ml sonicated nonhomologous DNA from herring sperm, and 11-DIG-UTP–labeled antisense cRNA probes. The DIG-labeled hybrids were detected by an enzyme-linked immunoassay using an anti-DIG-alkaline-phosphatase antibody conjugate. The subsequent enzyme-catalyzed chemiluminescence detection with the substrate AMPPD [3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphonyloxy)phenyl-1,2-dioxtane] visualized the hybrids on a light-sensitive film for quantification by densitometry with the National Institutes of Health Image 1.52 program (Bethesda, MD). Ribosomal bands were used as control markers for gel loading.

Western blot analyses. Pancreatic islets and other tissues were homogenized in ice-cold homogenization medium (20 mmol/l Hepes, 210 mmol/l mannitol, 70 mmol/l sucrose; pH 7.4). The tissue homogenates were centrifuged at 1,000*g* and 4°C for 10 min to pellet insoluble material. The supernatant was used for Western blot analyses. Protein was determined by the bicinchonic acid (BCA) assay (Pierce, Rockford, IL). Then 10 μg protein was fractionated by reducing 10% SDS polyacrylamide gel electrophoresis and electroblotted to polyvinylindene fluoride (PVDF) membranes. The membranes were stained by Ponceau to verify the transfer of comparable amounts of cellular protein. Nonspecific binding sites of the membranes were blocked by nonfat dry milk for 1 h at 37°C. The blots were then incubated with specific primary antibodies against SOD isoenzymes (21), catalase, Gpx (22), Hsp70, and heme oxygenase-1 at a dilution of 1:5,000–1:10,000 for 4 h at room temperature, followed by a 2-h incubation period with peroxidase-labeled secondary antibody at a dilution of 1:10,000 at room temperature. The protein bands were visualized by chemiluminescence using the ECL detection system.

Antioxidant enzyme activities. Organs of the rat were perfused with Krebs-Ringer bicarbonate buffer (KRBB; pH 7.4) through a catheter that was inserted into the abdominal aorta to remove contaminating blood. Tissues were homogenized in 50 mmol/l potassium phosphate buffer (pH 7.8). The homogenates were sonicated on ice for 1 min in 15-s bursts at 90 W with a Braun-Sonic 125 sonifier. Thereafter the homogenates were centrifuged at 35,000*g* and 4°C for 40 min, and the supernatant was stored at −20°C until measurement. SOD activities were measured in a photometric assay according to Oberley and Spitz (23) using xanthine oxidase as a source of O_2^- and NBT. The activities of the tissues were plotted against a standard curve of purified SOD from bovine liver. Addition of 5 mmol/l NaCN to the samples specifically inhibited Cu/Zn SOD. Subtraction of SOD activity after NaCN treatment from total SOD activity gave the Mn SOD activity of the sample. One unit of activity was defined as the amount of SOD protein that gives a half-maximal inhibition of NBT reduction. Catalase activity was measured by ultraviolet spectroscopy monitoring the decomposition of hydrogen peroxide at 240 nm (24). One unit of catalase activity was defined as μmol of hydrogen peroxide consumed per minute at 25°C. Gpx activity was measured in a photometric assay at 37°C using glutathione-reductase and NADPH in a coupled reaction (25). The decrease of NADPH absorbance was monitored at 340 nm. Activities were calculated against a Gpx standard according to Cornelius et al. (26) and expressed as U/mg protein.

Tissue culture of pancreatic islets and RINm5F cells. Freshly isolated pancreatic islets from rats and RINm5F insulinoma cells were cultured in RPMI 1640 medium, supplemented with 6.5 mmol/l glucose, 10% (vol/vol) fetal calf serum, penicillin, and streptomycin in a humidified atmosphere at 37°C and 5% CO_2. All experiments on RINm5F cells were performed with cells between passage 55 and 70. RINm5F cells were plated at a density of 1×10^6 per 90-mm plastic dish, and grown to confluency within 3 days. In experiments on isolated pancreatic islets, batches of 200–300 pancreatic islets were distributed to 90-mm plastic dishes with daily exchange of culture medium. Oxygen concentrations were kept constant in an incubator (Model 6000; Heraeus, Hanau, Germany) under control of an oxygen-sensitive electrode. Heat shock treatment of the cells was initiated by addition of prewarmed 41°C RPMI 1640 medium, followed by incubation at 41°C in a humidified incubator for 1 h. Thereafter the medium was replaced by culture medium prewarmed to 37°C, and the cells were incubated at 37°C for another 6 h to allow the expression of stress-response proteins. After this incubation period, the cells were prepared for Northern and Western blot analyses. The number of RINm5F cells was determined after trypsinization in a Neubauer counting chamber.

Overexpression of catalase and cytoplasmic Gpx in RINm5F cells. A full-length rat cytoplasmic Gpx cDNA (18) and a full-length human catalase cDNA (20) were subcloned into the pcDNA3 expression vector (Invitrogen, Leek, Netherlands) by standard molecular biology techniques (27,28). RINm5F insulinoma cells were transfected with the vector DNA by the use of lipofectamine (Gibco, Gaithersburg, MD). Positive clones were selected through resistance against G 418 (250 μg/ml) (Gibco) and verified by Northern blot analysis, Western blot analysis, and measurement of enzyme activity. Expression levels of both enzymes remained stable for at least 6 months. Because selenium is a cofactor for Gpx (29), this trace element (10 nmol/l) was included in the tissue culture medium of Gpx-transfected RINm5F cells. This increased the expression level by a factor of >10. Addition of selenium to control cells did not increase the Gpx expression.

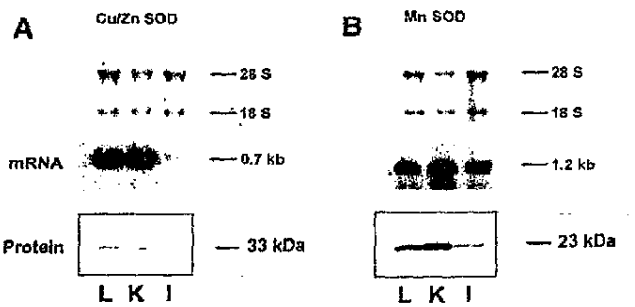

FIG. 1. Northern and Western blot analyses of Cu/Zn and Mn SOD expression in liver (*L*), kidney (*K*), and pancreatic islets (*I*) from rats. For Northern blot analysis, 5 μg total RNA were loaded per lane. mRNA levels were related to ribosomal bands and visualized in an ethidium bromide–stained agarose gel. Blots were probed with antisense cRNAs coding for rat cytoplasmic Cu/Zn SOD (*A*) and rat mitochondrial Mn SOD (*B*) by nonradioactive hybridization. For Western blot analysis, 10 μg protein from cellular homogenates were loaded per lane. Blots were probed with anti-rat Cu/Zn SOD (*A*) and anti-rat mitochondrial Mn SOD (*B*) antibodies and visualized by chemiluminescence. Shown are representative blots of at least four independent experiments.

Degradation of H_2O_2. After 24 hours, 4×10^4 cells were grown for 24 h in a 96-well dish and thereafter incubated with 100 μl 200 μmol/l H_2O_2 in KRBB at 37°C for 60 min. After 0, 5, 10, 20, 40 and 60 min, 50 μl aliquots of the medium were removed and mixed with 50 μl test buffer consisting of 300 mmol/l citric acid, 130 mmol/l Na_2HPO_4, and 0.75 μmol/l *O*-phenylenediamine at pH 5. The reaction was started by addition of 50 μl substrate solution consisting of 100 mU/ml horseradish peroxidase. After a 30-min incubation, the reaction was stopped by addition of 50 μl 0.5 mol/l H_2SO_4, and the absorbance of the colored reaction product was measured at 492 nm in a microplate reader.

Cytotoxicity tests. Catalase-transfected, Gpx-transfected, and, for comparison, nontransfected RINm5F insulin-producing cells were exposed at 37°C to hydrogen peroxide for 2 h in KRBB. Thereafter the buffer was removed and the cells were incubated for another 24 h in RPMI 1640 medium without hydrogen peroxide. The sensitivity of the cells to hydrogen peroxide toxicity was evaluated using a microtiter plate–based dimethylthiazoldiphenyl tetrazolium bromide (MTT) test (30) and lactate dehydrogenase (LDH) release into the medium (31).

Statistical analyses. The experimental data are expressed as means ± SE. Statistical analyses were performed using Student's *t* test. Linear regression analyses were performed by the least square method using the Prism analysis program (Graphpad, San Diego, CA). Linear associations between variables were expressed by the correlation coefficient *r*. The *P* value of the null hypothesis that the overall slope of the regression line is zero was calculated by the *F* test.

RESULTS

Expression of antioxidant enzymes in insulin-producing cells. The SOD isoenzymes were expressed in all tissues on the level of the mRNA, enzyme protein, and enzyme activity (Fig. 1 and Table 1). Cu/Zn SOD expression level in all tissues was in the range of at least 50% of that in the liver, whereas Mn SOD expression level was even higher in many tissues than in the liver (Table 1). Only in pancreatic islets

TABLE 1
Cu/Zn and Mn SOD gene and protein expression and enzyme activities in various rat tissues

Tissues	Enzyme	mRNA (% of liver)	Protein (% of liver)	Enzyme activity (% of liver)
Liver	Cu/Zn SOD	100 ± 3	100 ± 4	100 ± 7
	Mn SOD	100 ± 26	100 ± 0	100 ± 12
Kidney	Cu/Zn SOD	143 ± 9	91 ± 4	104 ± 10
	Mn SOD	238 ± 33	144 ± 12	105 ± 14
Spleen	Cu/Zn SOD	66 ± 8	40 ± 7	43 ± 5
	Mn SOD	141 ± 15	50 ± 6	25 ± 3
Lung	Cu/Zn SOD	67 ± 5	60 ± 7	49 ± 3
	Mn SOD	135 ± 7	84 ± 4	23 ± 4
Skeletal muscle	Cu/Zn SOD	52 ± 15	59 ± 9	42 ± 5
	Mn SOD	167 ± 22	80 ± 5	50 ± 6
Adipose tissue	Cu/Zn SOD	66 ± 4	36 ± 9	49 ± 5
	Mn SOD	207 ± 36	50 ± 9	84 ± 14
Brain	Cu/Zn SOD	73 ± 6	64 ± 5	37 ± 4
	Mn SOD	191 ± 26	136 ± 7	47 ± 7
Heart muscle	Cu/Zn SOD	86 ± 12	66 ± 7	74 ± 7
	Mn SOD	275 ± 43	160 ± 17	157 ± 21
Intestine	Cu/Zn SOD	51 ± 5	40 ± 4	40 ± 4
	Mn SOD	282 ± 33	74 ± 12	93 ± 9
Adrenal gland	Cu/Zn SOD	72 ± 10	43 ± 5	69 ± 12
	Mn SOD	141 ± 57	224 ± 21	275 ± 36
Pituitary gland	Cu/Zn SOD	58 ± 9	50 ± 5	141 ± 7
	Mn SOD	239 ± 33	104 ± 13	148 ± 8
Pancreatic islets	Cu/Zn SOD	23 ± 5	22 ± 5	31 ± 3
	Mn SOD	55 ± 6	38 ± 5	25 ± 2
RINm5F cells	Cu/Zn SOD	41 ± 8	57 ± 2	49 ± 2
	Mn SOD	47 ± 7	58 ± 9	60 ± 5

Data are means ± SE. RNA (5 µg) from each tissue was hybridized with antisense cRNA probes coding for rat cytoplasmic Cu/Zn SOD and rat mitochondrial Mn SOD. Protein (10 µg) from each tissue homogenate was subjected to Western blot analyses using antibodies against rat Cu/Zn and rat Mn SOD. Enzyme activities were determined in 35,000-g supernatants from sonicated tissue homogenates. One hundred percent enzyme activity in liver corresponds to 511 ± 37 U/mg protein for Cu/Zn SOD and 186 ± 23 U/mg protein for Mn SOD. The number of experiments was four to seven for each mean value.

were the levels of SOD isoenzyme expression well below 50% of those in liver (Fig. 1 and Table 1). In RINm5F insulinoma cells, the SOD isoenzyme expression levels were higher than in pancreatic islets (Table 1).

The expression of the hydrogen peroxide–inactivating enzymes catalase and cytoplasmic Gpx was highest in liver and kidney; lower in spleen, lung, adipose tissue, heart muscle and adrenal gland; and particularly low in skeletal muscle, brain, intestine, and pituitary gland (Fig. 2 and Table 2). The lowest expression levels, mostly less than 10% of those in the liver, were detected in pancreatic islets and RINm5F insulinoma cells (Table 2).

Membrane-bound phospholipid Gpx was expressed on the mRNA level in all tissues, with the exception of the pituitary gland (Table 3). Membrane-bound Gpx was not expressed in RINm5F insulinoma cells, indicating that the low level of expression of this enzyme in pancreatic islets may be accounted for by islet cell types other than the pancreatic β-cells. This may also explain the presence of Gpx enzyme activity in homogenates despite the absence of cytoplasmic Gpx mRNA and protein expression in pancreatic islets (Table 2).

The correlation between mRNA and protein levels as well as between protein levels and enzyme activities of the antioxidant enzymes revealed a significant association for Cu/Zn SOD, catalase, and Gpx, with correlation coefficients in the

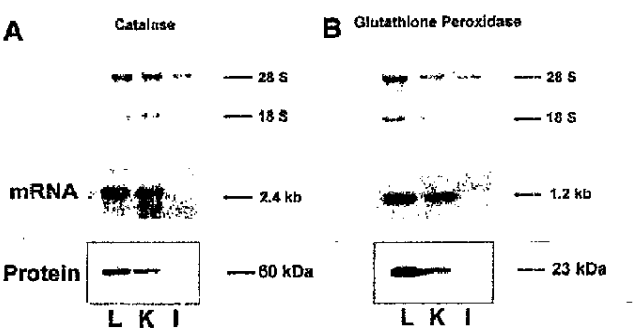

FIG. 2. Northern and Western blot analyses of catalase and cytoplasmic Gpx expression in liver (L), kidney (K), and pancreatic islets (I) from rats. For Northern blot analysis, 5 µg total RNA were loaded per lane. mRNA levels were related to ribosomal bands and visualized in an ethidium bromide–stained agarose gel. Blots were probed with antisense cRNAs coding for human catalase (*A*) and rat cytoplasmic Gpx (*B*) by nonradioactive hybridization. For Western blot analysis, 10 µg protein from cellular homogenates were loaded per lane. Blots were probed with anti-bovine catalase (*A*) and anti-rat cytoplasmic Gpx (*B*) antibodies and visualized by chemiluminescence. Shown are representative blots of at least four independent experiments.

TABLE 2
Catalase and cytoplasmic Gpx gene and protein expression and enzyme activities in various rat tissues

Tissues	Enzyme	mRNA (% of liver)	Protein (% of liver)	Enzyme activity (% of liver)
Liver	Catalase	100 ± 8	100 ± 0	100 ± 9
	Gpx	100 ± 3	100 ± 0	100 ± 8
Kidney	Catalase	109 ± 10	88 ± 4	64 ± 9
	Gpx	116 ± 28	79 ± 7	118 ± 7
Spleen	Catalase	55 ± 6	45 ± 4	19 ± 2
	Gpx	91 ± 16	65 ± 6	93 ± 11
Lung	Catalase	37 ± 4	35 ± 4	13 ± 2
	Gpx	66 ± 7	61 ± 2	67 ± 6
Skeletal muscle	Catalase	43 ± 8	13 ± 5	4 ± 1
	Gpx	59 ± 23	17 ± 3	31 ± 7
Adipose tissue	Catalase	84 ± 7	52 ± 16	32 ± 5
	Gpx	49 ± 10	23 ± 9	67 ± 7
Brain	Catalase	40 ± 9	10 ± 2	3 ± 0
	Gpx	15 ± 6	7 ± 3	11 ± 3
Heart muscle	Catalase	91 ± 11	22 ± 3	13 ± 1
	Gpx	122 ± 36	64 ± 7	115 ± 10
Intestine	Catalase	22 ± 2	26 ± 5	12 ± 4
	Gpx	20 ± 8	26 ± 2	29 ± 4
Adrenal gland	Catalase	56 ± 7	32 ± 4	10 ± 3
	Gpx	112 ± 28	68 ± 4	44 ± 7
Pituitary gland	Catalase	7 ± 2	10 ± 1	5 ± 1
	Gpx	32 ± 6	13 ± 7	51 ± 4
Pancreatic islets	Catalase	ND	ND	1 ± 0
	Gpx	ND	ND	21 ± 1
RINm5F cells	Catalase	13 ± 2	10 ± 1	2 ± 0
	Gpx	ND	ND	0 ± 1

Data are means ± SE. RNA (5 μg) from each tissue was hybridized with antisense cRNA probes coding for rat catalase and Gpx. Protein (10 μg) from each tissue homogenate was subjected to Western blot analyses using antibodies against bovine catalase and rat cytoplasmic Gpx. Enzyme activities were determined in 35,000-g supernatants from sonicated tissue homogenates. One hundred percent enzyme activity in liver corresponds to 324 ± 30 U/mg protein for catalase and 0.76 ± 0.06 U/mg protein for Gpx. The number of experiments was four to seven for each mean value. ND, not detectable.

0.8–0.9 range (Table 4). Only the association between Mn SOD mRNA and protein levels was weaker, with a correlation coefficient of 0.72, probably because of differences in the content of mitochondria in the examined tissues. Thus the level of gene expression of the antioxidant enzymes seems to determine the enzyme activity level in the various tissues.

Effects of cellular stress on antioxidant enzyme expression in pancreatic islets from rats

High glucose concentrations. Pancreatic islets can metabolize glucose at millimolar concentrations and use this enzymatic equipment to generate the signal for glucose-induced insulin secretion in its metabolism. The increase of oxidative metabolism is accompanied by the generation of free radicals that may induce antioxidant enzymes through a stress response mechanism. The increase of heme oxygenase-1 protein expression at 30 mmol/l glucose indicates that an increase in the glucose concentration may indeed initiate a stress response in pancreatic islets (Fig. 3). Nevertheless, incubation of isolated rat pancreatic islets with 30 mmol/l glucose for 48 h in tissue culture medium did not induce Cu/Zn and Mn SOD expression on either the level of mRNA or the protein or on the level of enzyme activities (Fig. 3 and Table 5). Catalase and Gpx mRNA and protein levels also remained below the detection limit after incubation of the islets with 30 mmol/l glucose (data not shown). Further, catalase and Gpx enzyme activities were not significantly increased by incubation with 30 mmol/l glucose (Table 5). RINm5F insulinoma cells, which, at variance with normal pancreatic β-cells, are not equipped enzymatically to increase glucose catabolism at glucose concentrations above 3 mmol/l, also did not react to 30 mmol/l glucose with changes of gene expression and enzyme activities of these antioxidant enzymes (data not shown).

High oxygen concentrations. A high oxygen concentration induces cellular stress through generation of reactive oxygen species. This was evident in the present experimental protocol from an increase of heme oxygenase-1 as a sensitive marker for the induction of cellular stress (Fig. 4). Incubation of isolated rat pancreatic islets in the presence of 70% O_2 for 48 h induced a marginally significant increase of Cu/Zn SOD mRNA expression, while the increase of protein expression was not significant (Fig. 4 and Table 6). Mn SOD mRNA and protein expression were not significantly increased in the presence of 70% O_2 (Fig. 4 and Table 6). In RINm5F insulinoma cells, the effects of a high oxygen concentration were even less pronounced (Table 6). Catalase and Gpx mRNA and protein expression in pancreatic islets as well as in RINm5F cells remained below the detection limit in Northern and Western blot analyses after a 1-h incubation in the presence of 70% O_2 (data not shown). Likewise, activity levels of both enzymes did not significantly increase in pancreatic islets or in RINm5F insulinoma cells (Table 6).

Heat shock treatment. A heat shock response was initiated

TABLE 3
Phospholipid hydroperoxide Gpx gene expression in various rat tissues

Tissues	mRNA (% of liver)
Liver	100
Kidney	140 ± 5
Spleen	49 ± 9
Lung	102 ± 12
Skeletal muscle	138 ± 18
Adipose tissue	94 ± 5
Brain	131 ± 6
Heart muscle	127 ± 13
Intestine	30 ± 10
Adrenal gland	114 ± 20
Pituitary gland	ND
Pancreatic islets	19 ± 5
RINm5F cells	ND

Data are means ± SE from four independent experiments. RNA (5 µg) from each tissue was hybridized with antisense cRNA probes coding for phospholipid hydroperoxide Gpx. ND, not detectable.

TABLE 4
Linear regression analyses of the relationship among antioxidant enzyme gene and protein expression and enzyme activity

	Cu/Zn SOD	Mn SOD	Catalase	Gpx
mRNA–enzyme protein	0.81*	0.58‡	0.92*	0.91*
Protein–enzyme activity	0.79†	0.82*	0.95*	0.89*

The linear association between the variables is expressed by the correlation coefficient r. The mean values of antioxidant enzyme gene expression, enzyme protein expression, and enzyme activities (all in percent of liver) from all tissues listed in Tables 1 and 2 were correlated between the variables mRNA/enzyme protein and enzyme protein/enzyme activity. In the case of the correlation between the variables mRNA/enzyme protein, the tissue adrenal gland proved to be an outlier with respect to the 95% CI so that this value was excluded from the calculation of the r value. Linear regression analyses were performed by the least square method. The P value of the null hypothesis that the overall slope of the regression line is zero was calculated from an F test. *$P < 0.001$; †$P < 0.002$; ‡$P < 0.05$.

through exposure of isolated rat pancreatic islets and RINm5F insulinoma cells to 41°C for 1 h. To allow expression of stress-response genes, the cells were incubated thereafter for another 6 h at 37°C. Under control conditions at 37°C Hsp70, protein expression was barely detectable in pancreatic islets and RINm5F cells. Heat shock treatment induced Hsp70 protein expression, but did not significantly affect Cu/Zn or Mn SOD expression (Fig. 5 and Table 7) or, indeed, catalase or Gpx expression (data not shown).

Overexpression of catalase and Gpx in RINm5F cells. Because both catalase and cytoplasmic Gpx expression were barely detectable in pancreatic β-cells and RINm5F insulinoma cells, and because higher expression could not be achieved through high glucose concentrations, high oxygen

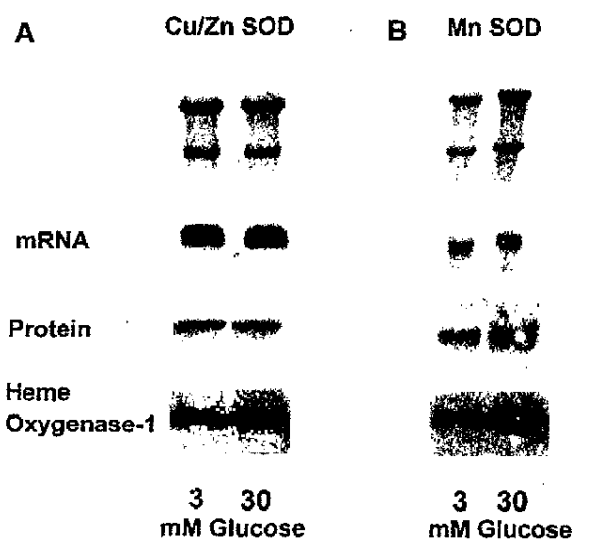

FIG. 3. Effects of a high glucose concentration (30 mmol/l) on SOD gene and protein expression in cultured rat pancreatic islets. Isolated rat pancreatic islets were incubated at 3 or 30 mmol/l glucose for 48 h. The 5 µg total RNA were analyzed by Northern blot hybridization using DIG-UTP–labeled cRNA probes coding for rat cytoplasmic Cu/Zn SOD (*A*) and rat mitochondrial Mn SOD (*B*). The 10 µg cellular protein were analyzed by immunoblotting using specific antibodies against rat Cu/Zn SOD (*A*) and rat Mn SOD (*B*). Induction of cellular stress was verified by immunoblotting of heme oxygenase-1 protein. Shown are representative blots of at least four independent experiments.

TABLE 5
Effects of a high glucose concentration (30 mmol/l) on antioxidant enzyme expression in rat pancreatic islets

	D-glucose (mmol/l)	
	3	30
Cu/Zn SOD		
mRNA	100 ± 11 (8)	89 ± 8 (9)
Protein	100 (7)	107 ± 9 (7)
Activity	100 ± 8 (5)	113 ± 5 (5)
Mn SOD		
mRNA	10(± 8 (7)	107 ± 9 (7)
Protein	100 (7)*	133 ± 9 (7)*
Activity	100 ± 7 (5)	125 ± 14 (5)
Catalase		
Activity	10(± 10 (4)	120 ± 8 (4)
Gpx		
Activity	100 ± 18 (5)	127 ± 14 (5)

Data are means ± SE (number of experiments). Rat pancreatic islets were incubated at 3 or 30 mmol/l glucose for 48 h. Gene expression was quantified by Northern blot hybridization, and protein expression was quantified by Western blot analyses using specific antibodies against rat Cu/Zn SOD and rat Mn SOD. Enzyme activities were measured by spectrophotometric assays. *$P < 0.05$ for 3 mmol/l vs. 30 mmol/l glucose.

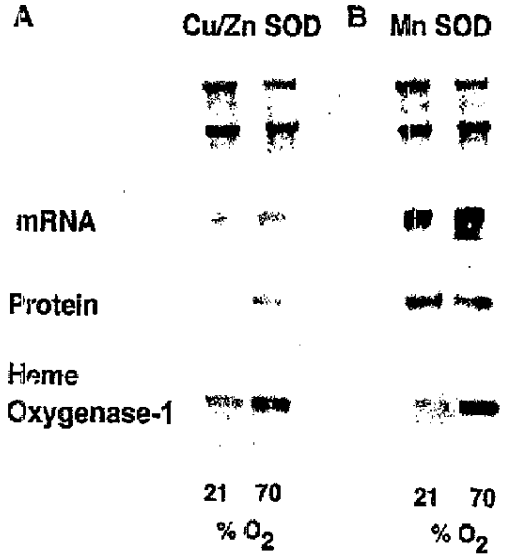

FIG. 4. Effects of high oxygen concentrations (70%) on SOD gene and protein expression in cultured pancreatic islets from rats. Isolated rat pancreatic islets were incubated at 21 or 70% O_2 in RPMI 1640 medium containing 5.5 mmol/l glucose for 48 h. The 5 µg total RNA were analyzed by Northern blot hybridization using DIG-UTP–labeled cRNA probes coding for rat cytoplasmic Cu/Zn SOD (*A*) and rat mitochondrial Mn SOD (*B*). The 10 µg cellular protein were analyzed by immunoblotting using specific antibodies against rat Cu/Zn SOD (*A*) and rat Mn SOD (*B*). Induction of cellular stress was verified by immunoblotting of heme oxygenase-1 protein. Shown are representative blots of at least four independent experiments.

concentrations, or heat shock treatment, attempts were made to overexpress both enzymes in RINm5F tissue culture cells. Through molecular biology techniques, we achieved a stable overexpression of both genes in RINm5F insulin-producing tumor cells (Fig. 6). We isolated three clones (Cat_{1-3}) transfected with human catalase cDNA and three clones (Gpx_{1-3}) transfected with rat Gpx cDNA with mRNA levels and enzyme protein levels higher than in rat liver (Fig. 6). The different transcript lengths of the mRNAs of the transfected clones are attributable to different polyadenylation activities of the bovine growth hormone gene at the 3′ end of the subcloned cDNA. As expected from the regression analysis of antioxidant enzyme expression, the clones with the highest mRNA expression also showed the highest protein expression and the highest enzyme activities. The enzyme activities were more than a 100-fold higher than in nontransfected control cells, and were also higher than in rat liver, which served as the reference tissue. Transfection did not affect insulin content, nor did it modify basal and KCl-induced insulin release of these RINm5F cells (data not shown).

Control RINm5F cells inactivated H_2O_2 (200 µmol/l) with a half-life of 19.5 ± 4.8 min (n = 4) (Fig. 7). The capacity for H_2O_2 inactivation was, however, significantly improved in catalase-transfected RINm5F cells with a half-life of 8.3 ± 2.0 min (n = 4) ($P < 0.05$), whereas the capacity of Gpx-transfected RINm5F cells was in the range of that of control cells, with a half-life of 24.3 ± 6.6 min (n = 4) (Fig. 7).

When compared with nontransfected control RINm5F cells, overexpression of catalase in RINm5F cells induced a significant increase in resistance toward H_2O_2 toxicity in both the MTT and LDH release assays (Figs. 8 and 9). Overexpression of Gpx in RINm5F cells provided a small but significant protection against H_2O_2 toxicity, whereas overexpression of catalase resulted in a drastic improvement of cellular defense. In the MTT test, the EC_{50} for H_2O_2 increased from 46 ± 1 µmol/l (n = 6) to 71 ± 3 µmol/l (n = 6) ($P < 0.05$

TABLE 6
Effects of a high oxygen concentration (70%) on antioxidant enzyme expression in cultured rat pancreatic islets and RINm5F insulinoma cells

	Expression (% of values at 21% O_2)			
	Pancreatic islets		RINm5F insulinoma cells	
	21% O_2	70% O_2	21% O_2	70% O_2
Cu/Zn SOD				
mRNA	100 (4)	141 ± 11 (4)*	100 (5)	98 ± 6 (5)
Protein	100 (4)	131 ± 10 (4)	100 ± 5 (4)	61 ± 12 (4)
Activity	100 ± 8 (5)	101 ± 10 (5)	100 ± 8 (5)	125 ± 9 (5)
Mn SOD				
mRNA	100 (4)	139 ± 18 (4)	100 (7)	112 ± 14 (7)
Protein	100 (4)	81 ± 10 (4)	100 ± 5 (4)	117 ± 7 (4)
Activity	100 ± 27 (5)	106 ± 26 (5)	100 ± 12 (4)	98 ± 14 (4)
Catalase				
Activity	100 ± 20 (5)	120 ± 40 (5)	100 ± 7 (4)	129 ± 13 (5)
Gpx				
Activity	100 ± 40 (5)	120 ± 22 (5)	100 ± 14 (4)	122 ± 13 (5)

Data are means ± SE (number of experiments). Rat pancreatic islets and RINm5F cells were cultured in RPMI 1640 medium containing 5.5 mmol/l glucose at either 21 or 70% O_2 for a period of 48 h. Gene expression was quantified by Northern blot hybridization, and protein expression was quantified by Western blot analyses using specific antibodies against rat Cu/Zn SOD and rat Mn SOD. Enzyme activities were measured by spectrophotometric assays. *$P < 0.05$ 21 vs. 70% O_2.

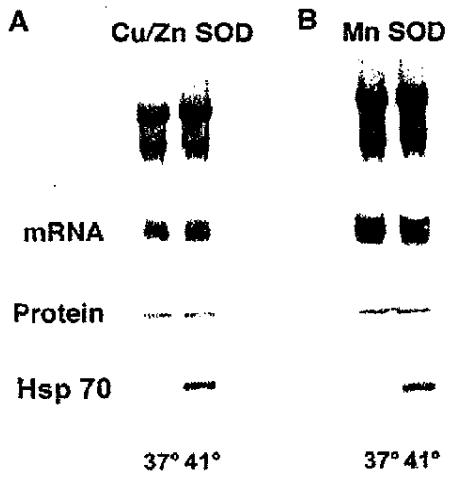

FIG. 5. Effects of heat shock treatment (41°C) on SOD gene and protein expression in cultured pancreatic islets from rats. Isolated rat islets were cultured in RPMI 1640 medium containing 5.5 mmol/l glucose. RINm5F cells were seeded at a density of 1 × 10^6 cells/9-cm dish and grown to confluency. Pancreatic islets and RINm5F cells were incubated at either 37 or 41°C for 1 h. Thereafter the cells were incubated for another 6 h at 37°C to allow expression of stress-response genes. The 5 µg total RNA were analyzed by Northern blot hybridization using DIG-UTP-labeled cRNA probes coding for rat cytoplasmic Cu/Zn SOD (*A*) and rat mitochondrial Mn SOD (*B*). The 10 µg cellular protein were analyzed by immunoblotting using specific antibodies against rat Cu/Zn SOD (*A*) and rat Mn SOD (*B*). For control of the stress response, Hsp70 protein expression was verified by immunoblot analysis by a specific anti Hsp70 antibody. Shown are representative blots of at least four independent experiments.

compared with nontransfected controls) for Gpx-transfected cells and to 592 ± 37 µmol/l (n = 6) ($P < 0.01$ compared with nontransfected controls) for catalase-transfected cells. In the LDH release test, the EC_{50} of H_2O_2 toxicity was 95 ± 19 µmol/l (n = 4) for nontransfected control cells and 96 ± 7 µmol/l (n = 4) for Gpx-transfected cells. Even at the highest concentration of 200 µmol/l H_2O_2, catalase-transfected cells showed only a small LDH release in the range of 5% of the total LDH content (Fig. 9). Calculation of an EC_{50} for H_2O_2 toxicity in catalase-transfected cells was not possible because higher concentrations of H_2O_2 destroyed the LDH enzyme activity (data not shown). Such an EC_{50} value would be well in the millimolar concentration range.

DISCUSSION

The present analysis of antioxidant enzyme expression in 12 different rat tissues showed that pancreatic islets and RINm5F insulin-producing tissue culture cells expressed SOD isoenzymes. The levels of the hydrogen peroxide–inactivating enzymes catalase and Gpx were, however, particularly low. This supports contentions from earlier studies (32–34) that indicated a low level of cytoprotective enzyme expression in pancreatic β-cells. Both enzymes were in a range approaching the lowest detection limit on the level of mRNA, enzyme protein, and enzyme activity, and thus were lower than in any other rat tissue studied.

Recent studies on isolated human pancreatic islets have indicated that these islets may be less susceptible to cytotoxic damage than rat islets (35). The authors detected up to twofold higher catalase activities in human than in rat islets, and interpreted this as an explanation for the lower susceptibility toward the toxic actions of streptozotocin and alloxan (36). However, doubling of the catalase enzyme activity in β-cells from 1 to 2% of the value in liver did not result in a hydrogen peroxide–inactivating capacity comparable with that found in other tissues (35,37). Other factors, such as the

TABLE 7
Effects of heat shock treatment on antioxidant enzyme expression in cultured pancreatic islets and RINm5F insulinoma cells

	Expression (% of values at 37°C)			
	Pancreatic islets		RINm5F insulinoma cells	
	37°C	41°C	37°C	41°C
Cu/Zn SOD				
mRNA	100 (4)	97 ± 13 (4)	100 (10)	129 ± 9 (8)
Protein	100 (9)	98 ± 3 (9)	100 (12)	101 ± 11 (4)
Activity	100 ± 15 (5)	100 ± 17 (5)	100 ± 9 (7)	105 ± 9 (7)
Mn SOD				
mRNA	100 (6)	93 ± 6 (6)	100 (12)	86 ± 8 (12)
Protein	100 (7)	99 ± 8 (7)	100 (9)	91 ± 11 (8)
Activity	100 ± 19 (5)	111 ± 13 (5)	100 ± 9 (6)	88 ± 12 (5)
Catalase				
Activity	100 ± 14 (5)	140 ± 29 (5)	100 ± 8 (5)	117 ± 13 (4)
Gpx				
Activity	100 ± 20 (4)	110 ± 18 (4)	100 ± 14 (4)	108 ± 12 (5)

Values are presented as means ± SE (number of experiments). Rat pancreatic islets and RINm5F cells were cultured in RPMI 1640 medium containing 5.5 mmol/l glucose at either 37 or 41°C for 1 h. Then the cells were incubated for further 6 h at 37°C to allow the expression of stress response genes. Gene expression was quantified by Northern blot hybridization, and protein expression was quantified by Western blot analyses using specific antibodies against rat Cu/Zn SOD and rat Mn SOD. Enzyme activities were measured by spectrophotometric assays.

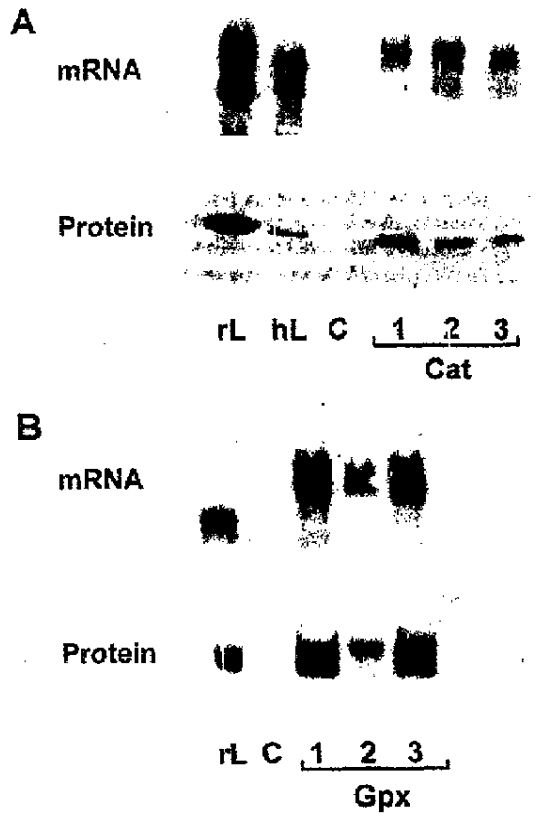

FIG. 6. Stable overexpression of (*A*) catalase and (*B*) cytoplasmic Gpx in RINm5F insulinoma cells. rL = rat liver; hL = human liver; C = nontransfected controls; 1–3 = G418 resistant clones. RINm5F cells were transfected with the human catalase cDNA and rat cytoplasmic Gpx cDNA. Positive clones Cat_{1-3} and Gpx_{1-3} were selected through resistance against G 418, and characterized for mRNA and protein expression as well as for catalytic activity. The catalase enzyme activities were for clone Cat_1, 564 ± 18 U/mg protein (n = 4); for clone Cat_2, 365 ± 9 U/mg protein (n = 4); and for clone Cat_3, 536 ± 5 U/mg protein (n = 3), with control 6 ± 1, U/mg protein (n = 4), and liver, 324 ± 30 U/mg protein (n = 6). The glutathione peroxidase enzyme activities were for clone Gpx_1, 4.35 ± 0.1 U/mg protein (n = 4); for clone Gpx_2, 0.77 ± 0.1 U/mg protein (n = 4); and for clone Gpx_3, 3.56 ± 0.2 U/mg protein (n = 4), with control, 0.04 ± 0.01 U/mg protein (n = 4), and liver, 0.76 ± 1.06 U/mg protein (n = 6).

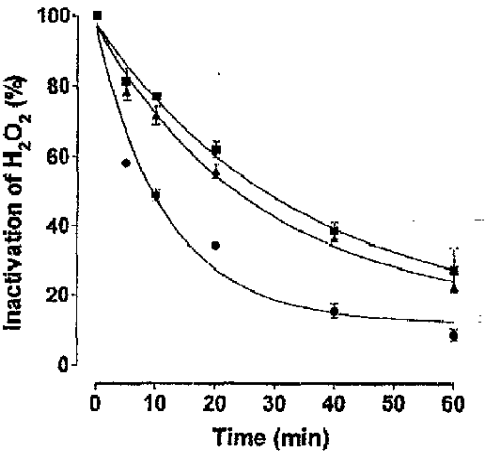

FIG. 7. Effects of stable overexpression of cytoplasmic glutathione peroxidase and catalase in RINm5F insulinoma cells on H_2O_2 degradation. ▲, nontransfected control cells; ■, Gpx-transfected cells; ●, catalase-transfected cells. Cells were exposed to 200 μmol/l for a period of 60 min at 37°C in KRBB. Aliquots of the medium were removed at time points 0, 5, 10, 20, 40, and 60 min for determination of H_2O_2 concentrations in a colorimetric assay. Data are given as means ± SE from four individual experiments and are expressed as a percentage of the concentration at 0 min.

higher expression level of protective Hsp70 (35) or the low abundance of GLUT2 in human β-cells, which is apparently used by these cytotoxic agents to reach intracellular space, may provide a better explanation for the lower susceptibility to streptozotocin and alloxan (38,39).

Parallel measurements of antioxidant enzyme mRNA and protein expression and enzyme activity in the present study enabled us to perform correlation analyses (Table 4) that strongly suggested that the antioxidant enzyme activities of the tissues are primarily determined by the level of mRNA. To elucidate the adaptive ability of antioxidant enzyme expression, we exposed pancreatic islets or RINm5F cells to conditions known to induce antioxidant enzymes significantly in other cellular systems.

High glucose concentrations have been reported to produce cellular stress in vascular endothelial cells and to increase SOD expression (40). Our data provided evidence that 30 mmol/l glucose also induces cellular stress in rat pancreatic islets, as indicated by a rise of heme oxygenase-1 protein expression in Western blot analyses, which serves as a sensitive marker of cellular stress (41,42). However, expression of SOD, catalase, and Gpx remained unaffected on the level of mRNA, protein, and enzyme activity. High oxygen concentrations that induced a marked increase of Mn SOD mRNA expression in alveolar cells from rats (43) did not induce this enzyme in rat pancreatic islets and RINm5F cells. Furthermore, heat shock treatment, a well-known inducer of cellular stress in insulin-producing cells (35), failed to enhance the expression of antioxidant enzymes in rat pancreatic islets and RINm5F cells. In conclusion, from our experiments on pancreatic β-cells as well as on RINm5F cells, various types of cellular stress, although verified by the induction of the stress marker proteins heme oxygenase-1 (41,42) and Hsp70 (35,44), did not induce any antioxidant enzyme on the level of mRNA, protein, or enzyme activity. Thus, unlike other tissues, insulin-producing cells apparently cannot adapt their level of antioxidant enzyme expression in response to these typical situations of cellular stress.

There are no reports in the literature on the ability of other stress inducers and toxic compounds to raise catalase or Gpx expression in insulin-producing cells to a significant extent. Only Mn SOD has been reported to show an increase in response to interleukin-1β (45,46). However, the abundance of the SOD isoenzymes is not particularly low in insulin-producing cells compared with other tissues (Table 1). Therefore we focused on the antioxidant enzymes with a low abundance in insulin-producing cells. The hydrogen peroxide–inactivating enzymes catalase and Gpx were overexpressed in RINm5F cells through stable transfection, yielding enzyme expression levels even higher than those in the liver.

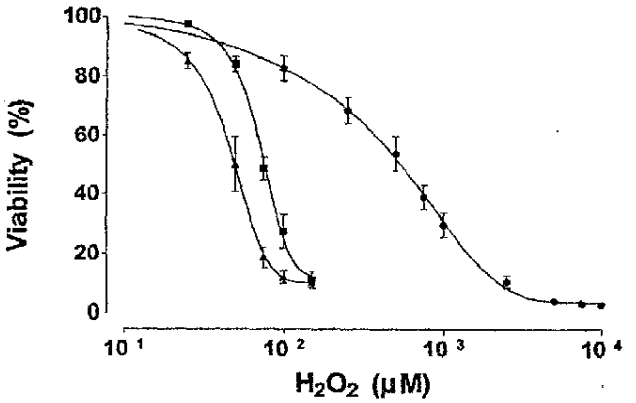

FIG. 8. Effects of stable overexpression of cytoplasmic glutathione peroxidase and catalase in RINm5F insulinoma cells on resistance against H_2O_2 in the MTT assay. ▲, nontransfected control cells; ■, Gpx-transfected cells; ●, catalase-transfected cells. Cells were exposed to serial concentrations of H_2O_2 for 2 h at 37°C in KRBB. Thereafter the cells were incubated for 24 h with RPMI 1640 medium. Viability was measured by the MTT assay and expressed in percentage of untreated controls. Data are given as means ± SE from six individual experiments.

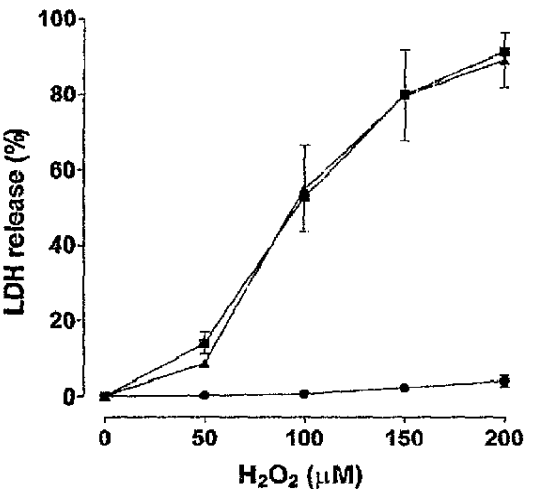

FIG. 9. Effects of stable overexpression of cytoplasmic glutathione peroxidase and catalase in RINm5F insulinoma cells on resistance against H_2O_2 in the LDH release assay. ▲, nontransfected control cells; ■, glutathione peroxidase-transfected cells; ●, catalase-transfected cells. Cells were exposed to various concentrations of H_2O_2 for 6 h at 37°C in KRBB. Thereafter LDH activity in the medium was measured by a colorimetric assay and expressed as percent of Triton X lysed cells without exposure to H_2O_2. Data are given as means ± SE from six individual experiments.

We subsequently assessed the role of these enzymes in protecting insulin-producing cells against hydrogen peroxide toxicity. Compared with control cells, a significant but small decrease of hydrogen peroxide toxicity was observed in the case of the Gpx-transfected RINm5F cells. Catalase overexpression, however, dramatically increased the resistance to hydrogen peroxide toxicity, as documented by an increase of the EC_{50} values more than 10-fold in the MTT test and the LDH release test. Thus inactivation of hydrogen peroxide seems to be a critical step in the removal of reactive oxygen species in insulin-producing cells.

In insulin-producing cells, the ratio of SOD enzymes to hydrogen peroxide inactivating enzymes is higher than in other tissues. This imbalance favors the accumulation of hydrogen peroxide. Hydrogen peroxide is a weak oxidant compared with the superoxide and hydroxyl radical. However, highly toxic hydroxyl radicals are formed from hydrogen peroxide by the Fenton reaction catalyzed by metals (10). Thus an induction of Mn SOD expression as reported for interleukin-1 in RINm5F insulin-producing tumor cells (45) and rat pancreatic β-cells (46) would not result in an improvement of the cellular defense status during autoimmune attack. Previous studies on Cu/Zn SOD–transfected and catalase-transfected mouse epidermal cells showed that SOD-overexpressing cells were hypersensitive to superoxide and hydrogen peroxide toxicity, whereas catalase-overexpressing cells were protected (47). These results imply that the ratio of SOD to catalase is more important for overall sensitivity than the level of SOD expression alone (47).

Another important aspect of hydrogen peroxide generation is its supposed role in signal transduction of cytokines activating transcriptional factors such as NF-κB (12,14). The importance of hydrogen peroxide in initiating signal cascades of β-cell destruction and protection is still controversial, and its precise role in activating genes controlling β-cell defense or destruction, and subsequently determining the fate of the β-cell, is not yet clear (8,48,49). Assuming that genes involved in β-cell damage (e.g., iNOS) do predominate during autoimmune attack, overexpression of catalase may be a way of blocking hazardous signal cascades leading to cell death. Through catalase overexpression, it may be possible to decrease the SOD/catalase enzyme activity ratio in pancreatic β-cells as a therapeutic concept, which would prevent the toxic action of many compounds that cause cellular damage through formation of hydrogen peroxide as a result of the SOD reaction.

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